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Development of Viremia and Humoral and Cellular Parameters of Immune Activation After Vaccination With Yellow Fever Virus Strain 17D: A Model of Human Flavivirus Infection

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To monitor early and late events of immune system activation after primary and secondary flavivirus infection, 17 healthy persons were vaccinated with the standard 17D vaccine virus strain of yellow fever (YF). Twelve of these persons had not received YF vaccine previously and 5 had been vaccinated once at least 10 years before. Viremia and various parameters of humoral and cellular immune activation were followed daily for 7 days and weekly thereafter. Viremia was detected by reverse transcriptase-polymerase chain reaction in all 12 first-time vaccinees beginning from the second to the sixth day after vaccination; most tested positive between the fourth and sixth day. Infectious 17D virus was detected using a plaque forming assay in the serum of 7 of the 12 first-time vaccinees. As first parameters of immune activation, neopterin and β2-microglobulin markedly increased between day 2 and day 6 postvaccination. In parallel to the viremia, circulating $CD8^+$ T-cells significantly increased, with peak levels at day 5 after primary vaccination, indicating an activation of the cellular immune system. Neither viremia nor significant changes of these activation markers were observed in the five revaccinated persons. Neutralizing antibodies directed against the 17D vaccine strain developed in all persons within 2 weeks after vaccination. No correlation was found between the extent of viremia and the titer of neutralizing antibodies. Revaccination was followed by a minor and transient increase of neutralizing antibodies. High titers of neutralizing antibodies persisted for at least 10 years after primary vaccination. ***J. Med. Virol. 56:159–167, 1998.*** © 1998 Wiley-Liss, Inc.

KEY WORDS: 17D viremia; neopterin; β2-microglobulin; $CD8^+$-T cells; neutralizing antibodies

Yellow fever (YF) remains a serious health problem in the endemic areas of tropical and subtropical Africa and South America. An effective and safe vaccine has existed since the introduction of the live, attenuated 17D vaccine strain against YF virus infection [Theiler and Smith, 1937]. Although the genomes of 17D substrains sequenced recently differ from the parental virulent Asibi virus strain in only 48 nucleotide changes scattered throughout the genome [dos Santos et al., 1995], the vaccine strain has markedly lower viscerotropism and neurotropism. Coincident with the augmented demand for YF vaccination resulting from increased tourism to endemic areas, the prevalence of immunodeficiency due to human immunodeficiency virus (HIV) infection among residents of industrialized countries has also increased. The use of live virus vaccines such as the 17D YF vaccine in immunocompromised persons has been debated extensively. Therefore, knowledge of the natural course of viremia and immune response after primary or repeated 17D YF vaccination is important. Because this knowledge is lacking even among immunocompetent persons, we studied the development of viremia and various specific and unspecific parameters of the cellular and humoral immune response in healthy persons after vaccination with the 17D YF live-virus strain.

Typically, natural viral infection and live virus vac-

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cination produces a strong and long lasting T-helper-cell-dependent neutralizing antibody response. Additionally, clearing of the intracellular pathogen via $CD8^+$ cytotoxic T lymphocytes (CTL) appears to be important for recovering from primary viral infection. For this reason, we monitored for 4 weeks after 17D YF vaccination $CD4^+$ and $CD8^+$ T-cell counts, soluble CD4-receptor (sCD4), and soluble interleukin-2 receptor (sIL-2) as markers of T-cell involvement, as well as β2-microglobulin and neopterin as indirect T-cell activation markers. The low molecular weight protein β2-microglobulin increases in body fluids after lymphocyte, especially T-cell, activation. The production and release of neopterin by monocyte/macrophages [Huber et al., 1984] is induced by interferon-γ (INFγ), which is released by activated T lymphocytes following viral infection. The capacity of vaccines to activate the cellular immune system and induce T-cell memory are important mechanisms of protection from wild-type infection, because T cells often recognize more conserved epitopes that do not change due to antibody mediated selection pressure [Bennink and Yewdell, 1988; Whitton and Oldstone, 1996]. Nevertheless, most vaccines licensed currently depend on neutralizing antibodies to control wild-type infections. For this reason, we studied the development of specific anti-17D antibodies and neutralizing antibodies against the 17D vaccine strain. To evaluate the relationship between antigenic challenge and immune response, viremia was monitored using a plaque forming assay (PA) and polymerase chain reaction (PCR).

MATERIAL AND METHODS

Study Design

Seventeen healthy persons from 18 to 50 years of age were vaccinated against YF at the World Health Organization (WHO)-registered vaccination center of the Robert Koch-Institute according to WHO international health regulations. Twelve persons, designated as first-time vaccinees, had not been vaccinated previously with the 17D vaccine nor had they had previous infection with the YF wild-type virus. Five persons, designated as revaccinees, had been vaccinated once with the 17D YF vaccine at least 10 years before. All 17 persons gave written consent after the purpose and design of the study was explained. Each participant received 0.5 ml vaccine subcutaneously. Blood samples were collected before vaccination, and 2, 3, 4, 5, and 6 days and 2 and 4 weeks postvaccination. Blood samples could be obtained from seven first-time vaccinees and five revaccinees 7 months after vaccination. The study participants were advised to report all clinical symptoms and side effects after vaccination. Viremia, T-cell subpopulations, β2-microglobulin, neopterin, tumor necrosis factor-α (TNFα), INFγ, liver enzymes, red and white blood cell counts, and differentials were measured.

17D YF Vaccine

The live, attenuated, WHO-approved, 17D YF vaccine (Lot No.: 134/94/10) used in this study was manufactured in avian leucosis-free chicken eggs according to WHO regulations at the Robert Koch-Institute, Berlin, Germany. A single vaccination dose contained approximately 4.8×10^4 plaque forming units (PFU).

Storage of Sera and Separation of Peripheral Blood Mononuclear Cells (PBMC)

All sera were stored in several aliquots at –70°C until use. The PBMCs were separated on a Ficoll-Hypaque gradient and processed immediately for flow cytometry (FACS).

PA for Detection of 17D Viremia

The PS-cell line (pig kidney cells), a line sensitive for flavivirus species, was used for detecting 17D viremia in a PA. The tests were carried out in 24-well plates (Nunc, Denmark) using a slightly modified technique described originally by De Madrid and Porterfield [1969]. PS-cells were cultured overnight at 37°C in Leibovitz medium (L-15, Gibco BRL, Germany) in a volume of 0.5 ml/well at a concentration of $3–4 \times 10^5$/ml. The cells were washed once with L-15 medium supplemented with 5% fetal calf serum (FCS) before adding 0.3 ml of a 1:5 dilution of the test sera to each of six wells in parallel. After 1 hr of incubation at 37°C, the sera were removed and 0.4 ml of L-15 medium supplemented with 5% FCS was added per well. The cells were overlaid with 1.6% carboxy-methyl-cellulose (CMC) sodium salt (BDH Chemicals Ltd., UK) in L-15 medium supplemented with 3% FCS. After 5 days of cultivation, the cell-layers were washed with phosphate-buffered saline (PBS) and fixed with 10% formaldehyde solution for 10 min. The fixed cells were stained with naphthalene black (Merck, Germany). The plaques were counted and the plaque forming units per milliliter (PFU/ml) were calculated.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Viral RNA was extracted by a modified form of the procedure described by Boom et al. [1990]. In brief, 50 μl of human serum were mixed with guanidine isothiocyanate lysis buffer and silica particles, fractionated by size. After incubation at room temperature for 10 min, the solution was vortexed and centrifuged. The silica nucleic acid pellet was washed twice with 1 ml of washing buffer, twice with 1 ml of 70% ethanol, and once with 1 ml of acetone. The pellet was allowed to dry in a heat block. The nucleic acids were eluted by adding 70 μl of Tris-ethylenediamine tetraacetic acid (EDTA) buffer to the pellet. The suspension was vortexed thoroughly and again incubated. After centrifugation, 50 μl of supernatant containing RNA was harvested.

All primers were chemically synthesized (TIB Molbiol Berlin, Germany). For first round amplification, a pair of published flavivirus type-specific primers was used [Eldahah et al., 1991]:

P1: 5′-TAC CCT GGA GCA AGA CAA GT -3′;
P2: 5′-GCT TTT CCA TAC CCA ATG AA-3′;

followed by a semi-nested PCR with a further, newly designed, specific downstream primer:

P3: 5′-CAC AAG TGA ATT TGG CG-3′.

All three primers were deduced from an RNA sequence from the envelope coding region of the YF virus. Viral RNA was converted to cDNA using reverse transcriptase and a specific YF virus downstream oligonucleotide primer (P1). The virus-specific cDNA was amplified by PCR using a second YF virus-specific, upstream oligonucleotide primer (P2) using a DNA polymerase kit (Biometra, Germany). Five microliters of the target RNA was first heat denatured (95°C for 1 min) and then amplified in 20 μl volumes containing the following components: 0.2 mM dNTP-mix, 0.1 M of each primer P1 and P2, 2 U of avian myeloblastosis virus reverse transcriptase (AMV RT, Serva, Heidelberg, Germany), 1 U of PrimeZyme DNA polymerase, and 1× reaction buffer. The reaction mixture was covered with mineral oil and incubated for RT for 50 min at 42°C. Thirty cycles of cDNA amplification (denaturing: 95°C for 1 sec, annealing: 53°C for 15 sec, extension: 72°C for 20 sec) were started immediately after RT, using a cycler equipped with modern peltier technology (Mastercycler 5330, Eppendorf, Germany).

To achieve optimum sensitivity, all relevant components of the RT-PCR including the temperature and time factor for annealing and extension as well as cell cycle number were initially optimized by the use of quantitated YF virus RNA. Using the cDNA products of the RT-PCR as templates for the nested PCR (N-PCR), primer P2 was replaced by nested primer P3. The amplified DNA fragments were visualized after agarose gel electrophoresis by ethidium bromide staining and UV light transillumination.

The sensitivity of the RT-PCR was determined by rediluting a lyophilized 17D virus stock (freeze dried at 1.15×10^5 PFU/ml) in human serum. The reliable limit of detectability of the RT-PCR/N-PCR was 1.15 PFU/ml.

Plaque Reduction Neutralization Test (PRNT)

The PRNT assays were carried out in 24-well plates (Nunc, Denmark). All test sera were assayed in quadruplicate. Two-fold dilutions of the test sera ranging from 1:10 to 1:320 were mixed with 100 tissue culture infectivity doses of the reference 17D virus preparation used in our laboratory (lot number: 354/1). After 1 hr of incubation at 37°C, an equal volume of PS-cell suspension containing 6×10^5 cells/ml was added and 0.4 ml of the mixture was plated on each of four wells. A 1.6% CMC/L-15 solution (BDH Chemicals Ltd., UK) supplemented with 3% FCS was overlaid after 4 hr of incubation. The cultures were maintained for 5 days at 37°C and then the cells were washed with PBS and fixed with 10% formaldehyde solution for 10 min. Naphthalene black was used for staining the cell-layers, plaques were counted, and the 90% neutralization titers were calculated according to Reed and Münch [1938].

Immunofluorescence Assay

17D-infected PS cells were grown for two days on glass slides with marked rings (Hölzel, Germany) at a density of 5×10^5/ml. Uninfected PS-cells were used as a negative control. After incubation in a humid chamber at 37°C, the cells were fixed for 30 min in ice cold acetone (Merck, Germany) and stored at –20°C. Immediately before usage, the slides were thawed at room temperature.

For specific anti-17D IgM detection, the IgG antibody fraction was removed from the test sera by incubation with anti-human IgG antibody according to the manufacturer's instructions (Mastsorb, Diagnostica, Germany). Before adding 20 μl of test serum per ring, the test sera were diluted two-fold with PBS supplemented with 2% bovine serum albumin (BSA; Sigma, Germany). Serum dilutions from 1:10 to 1:80 were analyzed. The slides were incubated in a humid chamber for 1 hr at 37°C and then washed three times with PBS and once with distilled water. After letting the slides air dry, 15 μl of an optimized dilution of a fluorescein isothiocyanate (FITC)-conjugated goat anti-human IgM or IgG antibody (Medac, Germany) were added per ring. The slides were incubated for 1 hr at 37°C and then washed three times in PBS. The slides were then evaluated using a fluorescence microscope (Zeiss, Germany). As a negative control, the uninfected PS cells on each slide were covered with 20 μl of a 1:10 dilution of the respective test serum. As positive control, a reference positive serum was included on every slide.

A similar procedure was used for the detection of antibodies cross-reacting with dengue virus type 1. Slides were coated as described above with Vero-B4 cells that were infected with dengue virus type 1 isolated from a patient from Nicaragua. The isolate was characterized by type-specific monoclonal antibodies (ATCC) and nested PCR.

FACS Analysis of Lymphocyte Subpopulations

After Ficoll-Hypaque purification, PBMC were washed twice in PBS. Selected concentrations of antibodies labeled directly were added to 2×10^5 PBMC. After incubation for 30 min at 4°C, cells were washed and resuspended in 0.4% formaldehyde. Appropriate irrelevant isotype-matched control antibodies and murine monoclonal antibodies directed against human CD8 (FITC) and CD4 Phyco-erythrin were used for labeling (Becton Dickinson, USA). Two-color flow cytometry was carried out with a FACScan flow cytometer and LYSIS II research software (Becton Dickinson). A total of 1×10^5 events/sample were collected. Erythrocytes, debris, and dead cells were excluded by gating according to forward and side scatter properties.

Analysis of Further Immunological and Clinical Parameters

Commercially available assays were used according to the manufacturers' instructions for the detection of

TABLE I. Course of Viremia After 17D-Yellow Fever Vaccination

Days after vaccination	Healthy, non-immune persons												Revaccinees				
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
2	3[a]	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
3	—[b]		—			—	—	—	—	—	—	—	—	—	—	—	—
4	—	—		83				—	—	—	—	—	—	—	—	—	—
5	—	—	—	97	3	6		3	3		—	—	—	—	—	—	—
6	—	—	—	8	17	6	6						—	—	—	—	—
13	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

[a]Shaded boxes indicate sera positive for circulating 17D viral RNA evaluated by reverse transcription-polymerase chain reaction (RT-PCR). The numbers within these boxes state the amount of 17D virus PFU/ml in serum in a plaque forming assay (PA) at the respective time points.
[b]— = Negative results in RT-PCR and PA.

β2-microglobulin, sIL-2 receptor (DRG Instruments GmbH, Marburg, Germany), sCD4-receptor (T-cell Diagnostics Inc., Cambridge, MA), neopterin (Brahms Diagnostica GmbH, Berlin, Germany), TNFα (Amersham Life Science, UK), and INFγ (Laboserv GmbH, Giessen, Germany). Aspartate aminotransferase, alanine aminotransferase, and γ-glutamyl transferase were determined with an Eppendorf Epos Analyzer 5060 (Eppendorf, Germany). Red and white blood cell and thrombocyte counts as well as differentials were undertaken using a Coulter-J.S. analyzer (Coulter, UK).

RESULTS

The 17D YF vaccination was well tolerated by all study participants and few side effects were reported or observed. Hyperemia at the site of injection occurred in 1 of 12 first-time vaccinees and in 2 of 5 revaccinees. No significant changes in the red and white blood cell and thrombocyte counts or the transaminases were detected after 17D YF vaccination.

Course of 17D-Viremia After Vaccination

Viremia after vaccination of the 17D vaccine strain was followed using RT-PCR, and for quantification, using classical PA. RT-PCR was significantly more sensitive than the PA. Starting as early as on day 2 following vaccination, circulating viral RNA was detected in the serum of all first-time vaccinees by RT-PCR. In 10 of the 12 first-time vaccinees, circulating viral RNA became detectable 4–6 days after vaccination (Table I). Circulating viable virus was detected in 7 of 12 first-time vaccinees using the PA. In 6 of the 7 PA-positive persons, the duration of viremia detected by RT-PCR surpassed that detected by the PA by 24 hr. Circulating virus was neither found by PA nor by RT-PCR at any time in the serum of the 5 revaccinees.

Neutralizing Antibodies

The occurrence of neutralizing antibodies (nAb) in serum after 17D vaccination was evaluated by the 90% plaque reduction assay. None of the first-time vaccinees showed preexisting nAb against the 17D virus strain. As early as 6 days after vaccination, low titers of nAb were detected in 3 of the 12 first-time vaccinees, and by 2 weeks after vaccination, nAb were detected in all 12 (Fig. 1). The mean nAb titer was 1:71 (SE ± 4.2) and 1:88 (SE ± 4.8) at 2 and 4 weeks postvaccination, respectively. After 7 months, nAb-levels remained unchanged at 1:75 (SE ± 4.6) among the 7 available first-time vaccinees. All revaccinees had persistent nAb before revaccination, with a mean titer of 1:72 (SE ± 11.2), despite an interval of at least 10 years since the last 17D vaccination. After revaccination, the nAb levels slightly increased to a mean titer of 1:169 (SE ± 34.4) and 1:126 (SE ± 11.4) at 2 and 4 weeks, respectively. Seven months after revaccination, the mean nAb titer of 1:88 (SE ± 13.4) was similar to that before revaccination. There was no correlation between the level and duration of detectable 17D viremia and the postvaccination nAb level.

Detection of 17D-Specific and Cross-Reacting IgM and IgG Antibodies

Before vaccination, specific anti-17D IgM or IgG antibodies (Ab) were not observed in the sera of the first-time vaccinees by immunofluorescence. Within 2 weeks after 17D YF vaccination, low levels of specific anti-17D IgM Ab were detected in 10 of 12 first-time vaccinees and in 4 of 5 revaccinees. Within 4 weeks after vaccination, all first-time vaccinees had developed pronounced specific anti-17D IgM Ab, with a mean titer of 1:58 (SE ± 11.9). At the same time, 11 of 12 first-time vaccinees also developed detectable specific anti-17D IgG Ab, with a mean titer of 1:33 (SE ± 12.2; data not shown).

Before revaccination, specific anti-17D IgG Ab were present in the serum of three of five revaccinees. Specific anti-17D IgM Ab, as expected, could not be demonstrated before boosting. Within 2 weeks after revaccination, four of the five revaccinees developed specific anti-17D IgM Ab and all had a rise in specific anti-17D IgG Ab (data not shown). Cross-reactivity of antibodies with another member of the flavivirus family, dengue virus type 1, was not common. None of the 12 first-time vaccinees had anti-dengue type 1 cross-reacting IgM Ab by immunofluorescence before or after 17D vaccination, and one developed de novo cross-reacting IgG Ab titers of 1:40 and >1:80, 2 and 4 weeks post 17D vaccination, respectively. One revaccinee, who had reported several trips to Southeast Asia, had a constant anti-dengue virus subtype 1 IgG titer of 1:20 before and after revaccination.

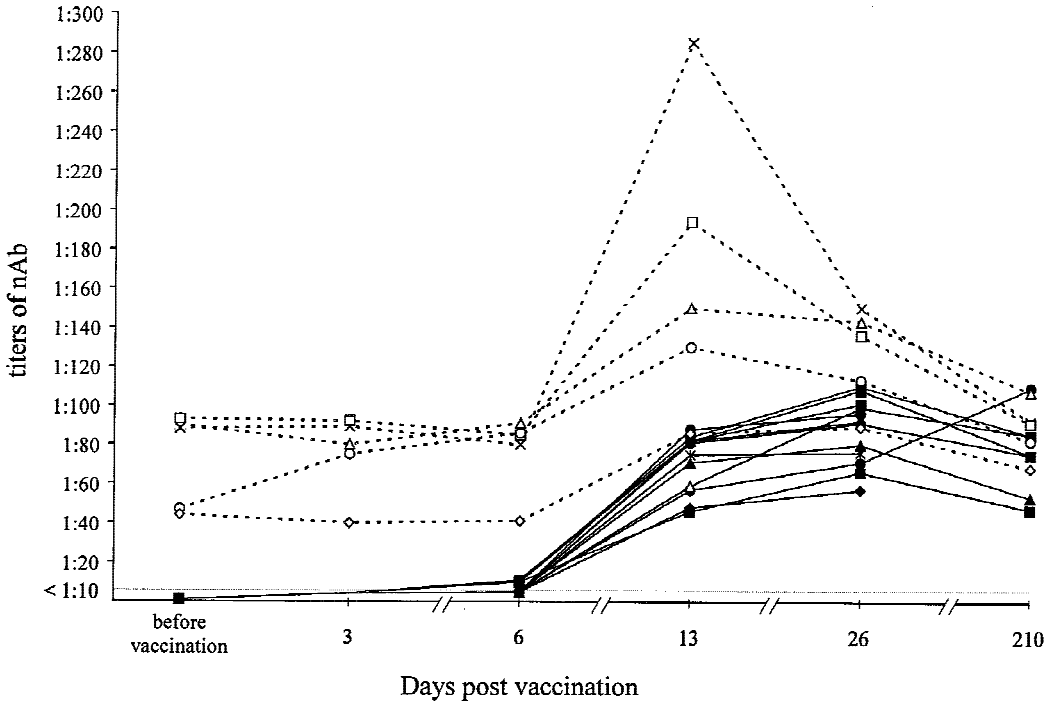

Fig. 1. The course of neutralizing antibodies (nAbs) following yellow fever (YF) vaccination. Reciprocal titers are shown. The continuous lines (–) represent the course of nAbs after primary vaccination ($n = 12$); the broken lines (---) represent the course of nAbs after revaccination ($n = 5$).

β2-Microglobulin and Neopterin After 17D Vaccination

β2-Microglobulin in serum increased in all first-time vaccinees after 17D vaccination; rising from a mean of 2.6 mg/l (SE ± 0.2 mg/l) before vaccination to 3.3 mg/l (SE ± 0.2 mg/l) on the third day postvaccination (Wilcoxon two sample test, $p = 0.0024$), which was in parallel with the detection of viremia. After the peak on the third postvaccination day, the β2-microglobulin levels in serum decreased slowly and by the 13th day postvaccination were at a level similar to those before vaccination (Fig. 2). A similar course was observed for neopterin, a molecule released by monocyte/macrophages after viral stimulation. The mean values increased from 6.5 nmol/l (SE ± 0.9 nmol/l) before immunization to a maximum of 10.8 nmol/l (SE ± 0.7 nmol/l) 3 days after immunization ($p = 0.0015$). Neopterin returned to prevaccination levels by the 13th postvaccination day (Fig. 2). Among the revaccinees, β2-microglobulin and neopterin levels did not change before revaccination (Fig. 2).

$CD8^+$ and $CD4^+$ T Cells and Cytokines After 17D Vaccination

Although absolute lymphocyte counts did not change significantly after vaccination, $CD8^+$ T cells increased because of a small increase in total lymphocyte count combined with a small initial decrease in $CD4^+$ T cells in all first-time vaccinees, which paralleled the 17D viremia. The level of circulating $CD8^+$ T cells increased from 22.7% (SE ± 1.6%) of the mononuclear cells on the day of vaccination to 32.9% (SE ± 1.8%) on the fourth postvaccination day (Fig. 3). This observed increase of $CD8^+$ T cells was highly significant ($p = 0.005$) among the first-time vaccinees, but among the revaccinees, did not change after revaccination. $CD4^+$ T cells did not significantly change after first-time vaccination or revaccination (data not shown). A slight initial decrease of $CD4^+$ T cells during the peak of viremia on the fourth and fifth day postvaccination was followed by a slight increase 2 weeks after vaccination, when the presence of specific nAb could be demonstrated in all vaccinees.

As first parameters of immune response to the 17D virus, a marked increase of β2-microglobulin and neopterin levels could be observed. $CD8^+$ T cells increased in parallel to the viremia and peaked shortly after circulating virus could no longer be detected.

Using a commercially available enzyme-linked immunosorbent assay (ELISA), INFγ or TNFα were not detected in the sera of any person at any time after vaccination. Furthermore, no significant changes could be detected in the course of sIL-2 or sCD4.

DISCUSSION

We analyzed the course of various parameters of the cellular and humoral immune response in relation to the course of viremia after administration of a single dose of commercially available 17D YF live vaccine. The development of the $CD8^+$ cellular immune response and related parameters were noted, since HLA-

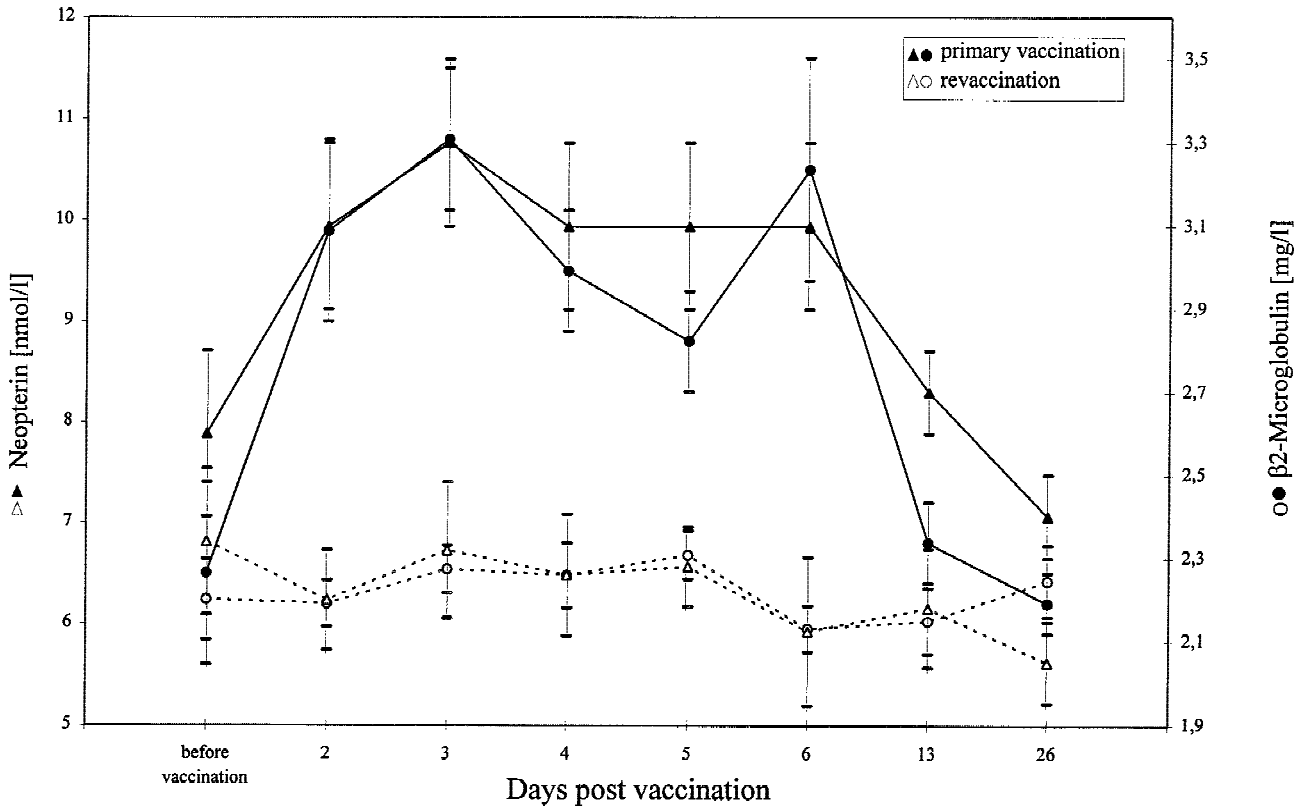

Fig. 2. Neopterin and β2-microglobulin levels after first-time and revaccination with 17D virus. The continuous line (–) represents the course of neopterin (▲, nmol/l) and β2-microglobulin (●, mg/l) after primary vaccination (n = 12); the broken lines (---) represent the course of neopterin (△) and β2-microglobulin (○) after revaccination (n = 5). Mean values (±SE) are presented.

class-l-restricted $CD8^+$ cytotoxic T lymphocytes are involved in controlling primary viral infections and primary challenges with attenuated live virus vaccines. To our knowledge, no data showing the parallel course of viremia and various parameters of the cellular and humoral immune responses in a single cohort after 17D vaccination have been reported.

To monitor viremia after 17D vaccination, results obtained by the PA were compared with modern molecular biological techniques. In agreement with results published previously [Wheelock and Sibley, 1965], the PA demonstrated small quantities of circulating infectious virus in 6 of 12 first-time vaccinees. However, the sensitivity of detection of viremia was substantially greater using RT-PCR. Circulating viral RNA was observed in all 12 first-time vaccinees and the duration of detectable viremia was at least 24 hr longer with RT-PCR compared with PA. Because RT-PCR detects stretches of virus-specific RNA that may represent only RNA fragments or defective viral particles, a positive assay does not necessarily reflect the presence of viable virus. In contrast, the formation of cytotoxic plaques in the PA indicates the presence of viable and infectious virus, a factor which probably contributed to the difference in sensitivity of the two assays. Among the revaccinees, neither circulating viral RNA nor viable virus could be detected, a finding similar to that reported by Wisseman and Sweet [1962]. The persistent nAbs titers in all of the revaccinees suggest that the proliferation of the 17D YF virus, and therefore detectable viremia, can be suppressed by their presence. Consistent with these findings, studies in rhesus monkeys and mice have shown that detectable viremia and clinical symptoms can be prevented by transferring antibodies passively before a wild-type virus challenge [Davis, 1934; Gould et al., 1986; Schlesinger et al., 1986].

None of the revaccinees had travelled to YF-endemic areas within 10 years of revaccination, yet, consistent with previously published results, all had high nAbs titers [Groot and Ribeiro, 1962; Poland et al., 1981]. These persistently high nAbs titers might be explained by the persistence of viral antigen [Gray and Matzinger, 1991; Gray et al., 1991; Oehen et al., 1992], most likely in local lymphatic tissue, as has been demonstrated for other arbovirus infections [Reeves et al., 1958]. Despite the absence of viremia, all revaccinees demonstrated a transient rise in nAb titers, indicating a specific immune response of memory B cells after rechallenge. It is possible that viremia was not detected because the methods used for detecting circulating virus were not sufficiently sensitive, particularly in the presence of high levels of nAbs. Seven months after revaccination, the nAb titers had returned to pre-boosting levels.

The 17D YF vaccine is known to be a safe and efficient vaccine. Accordingly, the development of nAbs

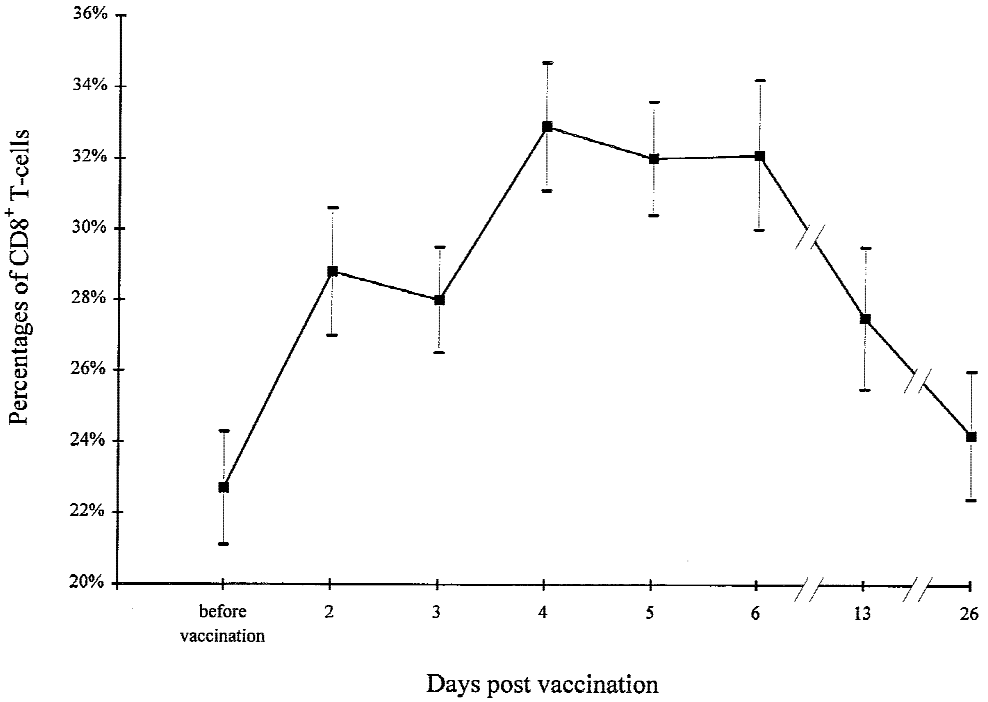

Fig. 3. Development of CD8+ T-cell counts in peripheral blood after first-time 17D yellow fever (YF) vaccination. The mean percentage of CD8+ T-cells of peripheral blood mononuclear cells (PBMC) (±SE) are shown ($n = 12$).

was demonstrated among the first-time vaccinees, indicating the vaccine's success. Seven months after primary challenge, nAbs titers were similar to those observed in the revaccinees, suggesting that nAb levels remain at relatively constant levels many years after 17D YF vaccination. Therefore, our data along with those showing persistence of nAb levels for 30–35 years after vaccination [Groot and Ribeiro, 1962; Poland et al., 1981] suggest that a single inoculation of the 17D YF vaccine may provide protection from wild-type YF infection longer than the 10-year revaccination interval recommended by WHO for international travel to YF-endemic areas.

Because the incidence of dengue fever epidemics is increasing worldwide, especially in South America, Southeast Asia, and the Caribbean, it is important to know the extent of anti-17D antibodies cross-reacting with dengue virus. To analyze the cross-reactivity of anti-YF antibodies with the dengue virus, the sera of all test persons were evaluated with an immunofluorescence assay. Only one study participant who had received a primary 17D vaccination, developed de novo anti-dengue virus subtype 1 cross-reacting IgG Ab. However, this person did not produce cross-reacting anti-dengue IgM Ab, which can be observed normally during or shortly after an acute dengue virus infection. Recently, another ubiquitous, clinically important member of the flavivirus family, the hepatitis C virus (HCV), was identified and several diagnostic assays were developed. Random serum samples of the first-time vaccinees and the revaccinees were tested by an HCV-ELISA. No cross-reacting antibodies against HCV were observed after 17D vaccination (E. Schreier/RKI, personal communication, data not presented). The data showing that the antibody response elicited after 17D YF vaccination is highly specific, with few persons developing cross-reacting antibodies, confirm earlier data obtained by other diagnostic methods [Rosenzweig et al., 1963; Yoshida et al., 1992].

Although humoral immunity involving CD4+ T-cell and B-cell activation and interaction is believed to play the major role in host defense against flavivirus infections [Monath, 1986], the importance of cell-mediated immunity, especially class I-restricted CD8+ T cells, in controlling primary flavivirus infections should not be underestimated. It is well known that a CTL response is crucial for complete recovery from viral infection and for induction of immunity following exposure to various viral infections and following attenuated, live-virus vaccination [Doherty and Zinkernagel, 1975]. Monkeys are protected from Asibi wild-type infection beginning the fifth day after vaccination with the 17D YF vaccine, at a time point when nAb are still undetectable [Smithburn and Mahaffy, 1945; Theiler and Smith, 1937]. In 1992, Hill demonstrated a specific CTL response in humans and mice following flavivirus infection [Hill et al., 1992]. Additionally, Rothman et al. [1993] described the recognition of proteins isolated from dengue virus by specific CD8+ CTL clones.

So far, few data exist elucidating the involvement of CD8+ T cells in the immune response after 17D live-virus vaccination. Although we did not measure 17D-specific CTL, the course of circulating CD8+ T-cell counts following viral challenge was monitored. Among

the first-time vaccinees, a significant increase was observed in the $CD8^+$ T-cell population, a finding that strongly suggested that $CD8^+$ T cells were involved in the primary immune reaction to YF vaccination. Levels of $CD8^+$ T cells and of circulating virus peaked simultaneously. Furthermore, $CD8^+$ T cells preceded the production of nAbs by several days, suggesting that $CD8^+$ T cells were important in the control of viremia after 17D YF vaccination. It has been shown that with the development of the $CD8^+$ cytotoxic T-cell response, viruses were eliminated within 7–10 days of primary infection and after this time, viral genomic material was no longer detected [Eichelberger et al., 1991]. In our group of revaccinees, persistent nAb were present, preventing potentially detectable viremia. Therefore, a sufficient antigenic stimulus to induce a significant rise in $CD8^+$ T cells may not have existed after revaccination. Whether or not 17D-specific $CD8^+$ CTL memory cells are stimulated by revaccination is subject to further study.

In addition to monitoring $CD8^+$ T cells, we followed the course of β2-microglobulin after vaccination. Extensive evidence indicates that lymphocytes are the main source of β2-microglobulin and the activation of lymphocytes expressing a high density of major histocompatibility complex (MHC) class-I molecules, such as occurs during viral infection or as a result of an immune response to tumors, is associated with an increased release of this protein. In contrast to previously published results [Roers et al., 1994], it was observed that after first-time 17D YF vaccination β2-microglobulin increased in parallel with circulating $CD8^+$ T cells, suggesting that this cell population was activated after primary vaccination. A similar relationship was noted with neopterin levels following first-time vaccination. Most likely the increase in neopterin, which is produced mainly by monocyte/macrophages, was triggered by INFγ released by activated T lymphocytes following the 17D viral challenge. In our study, however, INFγ levels remained below detectable limits, possibly due to the short half-life of this cytokine or due to the insufficient sensitivity of the assay system used.

It is concluded that following primary immunization with the 17D vaccine, there is an early and viremia-dependent involvement of the cellular immune system, especially by $CD8^+$ T cells, as well as an efficient induction of long lasting neutralizing antibodies. The basic immunobiological data derived from this study, conducted in healthy volunteers, provides a basis for speculation about the immunological reactions following 17D live virus vaccination in immunocompromised persons.

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